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(54) RECOMBINANT DNA EXPRESSION VECTORS.

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JOURNAL OF VIROLOGY, vol. 56, no. 1, October 1985, American Society for Microbiology (US); R.R.SPAETE et al., pp. 135-143*

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DescriptionField of the Invention

5 This invention relates to expression vectors containing a DNA sequence from the human cytomegalovirus major immediate early gene, to host cells containing such vectors, to a method of producing a desired polypeptide by using vectors containing said sequence and to the use of said DNA sequence.

10 Background to the Invention

The main aim of workers in the field of recombinant DNA technology is to achieve as high a level of production as possible of a particular polypeptide. This is particularly true of commercial organisations who wish to exploit the use of recombinant DNA technology to produce polypeptides which naturally are not

15 very abundant. Generally the application of DNA technology involves the cloning of a gene encoding the desired polypeptide, placing the cloned gene in a suitable expression vector, transfecting a host cell line with the vector, and culturing the transfected cell line to produce the polypeptide. It is almost impossible to predict whether any particular vector or cell line or combination thereof will lead to a useful level of production.

20 In general, the factors which significantly affect the amount of polypeptide produced by a transfected cell line are: 1. gene copy number, 2. efficiency with which the gene is transcribed and the mRNA translated, 3. the stability of the mRNA and 4. the efficiency of secretion of the protein.

The majority of work directed at increasing expression levels of recombinant polypeptides has focussed on improving transcription initiation mechanisms. As a result the factors affecting efficient translation are

25 much less well understood and defined, and generally it is not possible to predict whether any particular DNA sequences will be of use in obtaining efficient translation. Attempts at investigating translation have consisted largely of varying the DNA sequence around the consensus translation start signal to determine what effect this has on translation initiation (Kozak M. Cell 41 283-292 (1986)).

30 Studies involving expression of desired heterologous genes normally use both the coding sequence and at least part of the 5'-untranslated sequence of the heterologous gene such that translation initiation is from the natural sequence of the gene. This approach has been found to be unreliable probably as a result of the 'hybrid nature' of the 5'-untranslated region and the fact that the presence of particular 5'-untranslated sequences can lead to poor initiation of translation (Kozak M. Procl. Natl. Acad. Sci. 83 2850-2854 (1986) and Pelletier and Sonenberg Cell 40 515-526 (1985)). This variation in translation has a detrimental effect on the amount of the product produced.

35 Previous studies (Boshart et al Cell 41 521-530 (1985) and Pasleau et al, Gene 38 227-232 (1985); Stenberg et al, J. Virol 49 (1) 190-199 (1984); Thomsen et al Proc. Natl. Acad. Sci. USA 81 659-663 (1984) and Foecking and Hofstetter Gene 45 101-105 (1986)) have used sequences from the upstream region of the hCMV-MIE gene in expression vectors. These have, however, solely been concerned with the use of the sequences as promoters and/or enhancers. Spaete and Mocarski (J. Virol 56 (1) 135-143, 1985) have used a PstI to PstI fragment of the hCMV-MIE gene encompassing the promoter, enhancer and part of the 5'-untranslated region, as a promoter for expression of heterologous genes. In order to obtain translation the natural 5'-untranslated region of the heterologous gene was used.

45 In published European Patent Application No. 260148, a method for the continuous production of a heterologous protein is described. The expression vectors constructed contain part of the 5'-untranslated region of the hCMV-MIE gene as a stabilising sequence. The stabilising sequence is placed in the 5'-untranslated region of the gene encoding the desired heterologous protein i.e. the teaching is again that the natural 5'-untranslated region of the gene is essential for translation.

50 Summary of the Invention

In a first aspect the invention provides a vector containing a DNA sequence comprising the promoter, enhancer and functionally complete 5'-untranslated region including the first intron of the human

55 cytomegalovirus major immediate early gene, wherein the 5'-untranslated region is not linked directly to the DNA coding sequence of the natural human cytomegalovirus major immediate early gene. In a preferred embodiment of the first aspect of the invention, the vector includes a restriction site for insertion of a heterologous gene.

The present invention is based on the discovery that vectors containing a DNA sequence comprising the promoter, enhancer and complete 5'-untranslated region of the major immediate early gene of the human cytomegalovirus (hCMV-MIE) upstream of a heterologous gene result in high level expression of the heterologous gene product. In particular, we have unexpectedly found that when the hCMV-MIE derived DNA is linked directly to the coding sequence of the heterologous gene high levels of mRNA translation are achieved. This efficient translation of mRNA is achieved consistently and appears to be independent of the particular heterologous gene being expressed.

In a second aspect the invention provides a vector containing a DNA sequence comprising the promoter, enhancer and functionally complete 5'-untranslated region including the first intron of the human cytomegalovirus major immediate early gene, wherein the 5'-untranslated region is linked directly to the DNA coding sequence of a heterologous gene.

The hCMV-MIE derived DNA according to the second aspect of the invention may be separated from the coding sequence of the heterologous gene by intervening DNA such as for example by the 5'-untranslated region of the heterologous gene.

Preferably the hCMV-MIE derived sequence includes a sequence identical to the natural hCMV-MIE translation initiation signal. It may however be necessary or convenient to modify the natural translation initiation signal to facilitate linking the coding sequence of the desired polypeptide to the hCMV-MIE sequence, i.e. by introducing a convenient restriction enzyme recognition site. For example the translation initiation site may advantageously be modified to provide an NcoI recognition site.

The heterologous gene may be a gene coding for any eukaryotic polypeptide such as for example a mammalian polypeptide such as an enzyme, e.g. chymosin or gastric lipase; an enzyme inhibitor, e.g. tissue inhibitor of metalloproteinase (TIMP); a hormone, e.g. growth hormone; a lymphokine, e.g. an interferon; a plasminogen activator, e.g. tissue plasminogen activator (tPA) or prourokinase; or a natural, modified or chimeric immunoglobulin or a fragment thereof including chimeric immunoglobulins having dual activity such as antibody-enzyme or antibody-toxin chimeras.

According to a third aspect of the invention there is provided host cells transfected with vectors according to the first or second aspect of the invention.

The host cell may be any eukaryotic cell such as for example plant, or insect cells but is preferably a mammalian cell such as for example CHO cells or cells of myeloid origin e.g. myeloma or hybridoma cells.

In a fourth aspect the invention provides a process for the production of a heterologous polypeptide by culturing a transfected cell according to the third aspect of the invention.

In a fifth aspect the invention provides the use of a DNA sequence comprising the promoter, enhancer and subsequently complete 5'-untranslated region including the first intron of the hCMV-MIE gene for expression a heterologous gene.

In a preferred embodiment of the fifth aspect of the invention the hCMV-MIE derived DNA sequence is linked directly to the DNA coding sequence of the heterologous gene.

Also included within the scope of the invention are plasmids pCMGS, pHT.1 and pEE6hCMV.

Brief Description of the Drawings

The present invention is now described, by way of example only, with reference to the accompanying drawings in which

Figure 1: shows a diagrammatic representation of plasmid pSVLGS.1

Figure 2: shows a diagrammatic representation of plasmid pHT.1

Figure 3: shows a diagrammatic representation of plasmid pCMGS

Figure 4: shows the complete sequence of the promoter-enhancer hCMV-MIE including both the first intron and a modified translation 'start' site

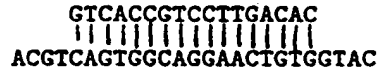
Figure 5: shows a diagrammatic representation of plasmid pEE6.hCMV

Detailed Description of the Embodiments

Example 1

The Pst-1m fragment of hCMV (Boshart et al Cell 41 521-530 (1985) Spaete & Mocarski J. Virol 56 (1) 135-143 (1985)) contains the promoter-enhancer and most of the 5'-untranslated leader of the MIE gene including the first intron. The remainder of the 5'-untranslated sequence can be recreated by attaching a small additional sequence of approximately 20 base pairs.

Many eukaryotic genes contain an NcoI restriction site (5'-CCATGG-3') overlapping the translation start site, since this sequence frequently forms part of a preferred translation initiation signal 5' ACCATG Pu-3'. The hCMV-MIE gene does not have an NcoI site at the beginning of the protein coding sequence but a single base-pair alteration causes the sequence both to resemble more closely the "Kozak" consensus initiation signal and introduces an NcoI recognition site. Therefore a pair of complementary oligonucleotides were synthesised of the sequence:



which when fused to the Pst-1m fragment of hCMV will recreate the complete 5'-untranslated sequence of the MIE gene with the single alteration of a G to a C at position -1 relative to the translation initiation codon.

This synthetic DNA fragment was introduced between the hCMV Pst-1m promoter-enhancer leader fragment and a glutamine synthetase (GS) coding sequence by ligation of the Pst-1m fragment and the synthetic oligomer with NcoI digested pSV2.GS to generate a new plasmid, pCMGS (The production of pSV2.GS is described in published International Patent Application No. WO 8704462). pCMGS is shown in Figure 3. pCMGS thus contains a hybrid transcription unit consisting of the following: the synthetic oligomer described above upstream of the hCMV-MIE promoter-enhancer (where it serves merely as a convenient PstI - NcoI "adaptor"), the hCMV-MIE promoter and the complete 5' untranslated region of the MIE gene, including the first intron, fused directly to the GS coding sequence at the translation initiation site.

pCMGS was introduced into CHO-K1 cells by calcium phosphate mediated transfection and the plasmid was tested for the ability to confer resistance to the GS-inhibitor methionine sulfoximine (MSX). The results of a comparison with pSV2.GS are shown in Table 1.

It is clear that pCMGS can confer resistance to 20 M MSX at a similar frequency to pSV2.GS, demonstrating that active GS enzyme is indeed expressed in this vector.

Table 1

Results of transfection of GS-expression vectors into CHO-K1 cells	
Vector	no. colonies/10 ⁶ cells resistant to 20μM MSX
pSV2.GS	32
pCMGS	17
Control	0

Example 2

The TIMP cDNA and SV40 polyadenylation signal as used in pTIMP 1 Docherty et al (1985) Nature 318, 66-69, was inserted into pEE6 between the unique HindIII and BamHI sites to create pEE6TIMP. pEE6 is a bacterial vector from which sequences inhibitory to replication in mammalian cells have been removed. It contains the XmnI to BclI portion of pCT54 (Emtage et al 1983 Proc. Natl. Acad. Sci. USA 80, 3671-3675) with a pSP64 (Melton et al 1984: Nucleic Acids. Res. 12, 7035) polylinker inserted in between the HindIII and EcoRI sites. The BamHI and Sall sites have been removed from the polylinker by digestion, filling in with Klenow enzyme and religation. The BclI to BamHI fragment is a 237 bp SV40 early polyadenylation signal (SV40 2770 to 2533). The BamHI to the BglI fragment is derived from pBR328 (375 to 2422) with an additional deletion between the Sall and the Aval sites (651 to 1425) following the addition of a Sall linker to the Aval site. The sequence from the BglI to the XmnI site originates from the β-lactamase gene of pSP64.

The 2129 base-pair NcoI fragment containing the hCMV MIE promoter-enhancer and 5' untranslated sequence was isolated from pCMGS by partial NcoI digestion and inserted at the NcoI site overlapping the translation initiation signal of TIMP in pEE6TIMP to generate the plasmid pHT.1 (shown in Figure 2).

A GS gene was introduced into pHT.1 to allow selection of permanent cell lines by introducing the 5.5K PvuI - BamHI fragment of pSVLGS.1 (figure 1) at the BamHI site of pHT.1 after addition of a synthetic BamHI linker to PvuI digested pSVLGS.1 to form pHT.1GS. In this plasmid the hCMV-TIMP and GS transcription units transcribe in the same orientation.

pHT.1 GS was introduced into CHO-K1 cells by calcium-phosphate mediated transfection and clones resistant to 20 μ M MSX were isolated 2-3 weeks post-transfection. TIMP secretion rates were determined by testing culture supernatants in a specific two site ELISA, based on a sheep anti TIMP polyclonal antibody as a capture antibody, a mouse TIMP monoclonal as the recognition antibody, binding of the monoclonal being revealed using a sheep anti mouse IgG peroxidase conjugate. Purified natural TIMP was used as a standard for calibration of the assay and all curves were linear in the range of 2 - 20ng ml⁻¹. No non-specific reaction was detectable in CHO-cell conditioned culture media.

One cell line GS.19 was subsequently recloned, and a sub-clone GS 19-12 secretes TIMP at a very high level of 3 x 10⁸ molecules/cell/day. Total genomic DNA extracted from this cell line was hybridised with a TIMP probe by Southern blot analysis using standard techniques and shown to contain a single intact copy of the TIMP transcription unit per cell (as well as two re-arranged plasmid bands). This cell line was selected for resistance to higher levels of MSX and in the first selection a pool of cells resistant to 500 μ M MSX was isolated and recloned. The clone GS-19.6(500)14 secretes 3 x 10⁹ molecules TIMP/cell/day. The vector copy-number in this cell line is approx. 20 - 30 copies/cell. Subsequent rounds of selection for further gene amplification did not lead to increased TIMP secretion.

Thus it appears that the hCMV-TIMP transcription unit from pHT.1 can be very efficiently expressed in CHO-K1 cells at approximately a single copy per cell and a single round of gene amplification leads to secretion rates which are maximal using current methods.

Example 3

In order to test whether the hCMV-MIE promoter-enhancer-leader can be used to direct the efficient expression of other protein sequences, two different but related plasminogen activator coding sequences (designated PA-1 and PA-2) were introduced into CHO-K1 cells in vectors in which the protein coding sequences were fused directly to the hCMV sequence.

In both these cases, there is no Nco1 site at the beginning of the translated sequence and so synthetic oligonucleotides were used to recreate the authentic coding sequence from suitable restriction sites within the translated region. The sequence of the modified hCMV translation-initiation signal as used in pHT.1 was also built into the synthetic oligonucleotide which then ended in a Pst-1 restriction site. The Pst-1m fragment of hCMV was then inserted at this site to create the complete promoter-enhancer-leader sequence.

The hCMV-plasminogen activator transcription units were introduced into CHO-K1 cells after inserting a GS gene at the unique BamHI site as above and MSX resistant cell lines secreting plasminogen activator were isolated.

The secretion rates of the best initial transfectant cell lines in each case are given in Table 2. From this it is clear that the hCMV promoter-enhancer leader can also be used to direct the efficient expression of these two plasminogen activator proteins.

Table 2

Secretion rates of the different plasminogen activator proteins from transfectant CHO cell lines.	
Plasminogen activator	Molecules secreted/cell/day
PA - 1	5.5 x 10 ⁷
PA - 2	1.1 x 10 ⁸

Example 4

pEE6hCMV was made by ligating the Pst-1m fragment of hCMV, HindIII -digested pEE6 and the complementary oligonucleotides of the sequence:

GTCACCGTCCTTGACACGA
 |||||
 ACGTCAGTGGCAGGAAGTGTGCTTCGA

cDNA encoding an immunoglobulin light-chain was inserted at the EcoRI site of pEE6.hCMV such that the hCMV-MIE promoter-enhancer leader could direct expression of the cDNA and a selectable marker gene containing the SV40 origin of replication was inserted at the BamHI site of each plasmid.

This plasmid was transfected into COS-1 monkey kidney cells by a standard DEAE-dextran transfection procedure and transient expression was monitored 72 hours post transfection. Light chain was secreted into the medium at at least 100ng/ml indicating that light chain can indeed be expressed from a transcription unit containing the entire hCMV-MIE 5'-untranslated sequence up to but not including the translation initiation ATG, followed by 15 bases of natural 5'-untranslated sequence of the mouse immunoglobulin light-chain gene.

Claims

1. A vector for use in eukaryotic cell lines containing a DNA sequence comprising the promoter, enhancer and functionally complete 5'-untranslated region including the first intron of the human cytomegalovirus major immediate-early gene, wherein the 5'-untranslated region is not linked directly to the DNA coding sequence of the natural human cytomegalovirus major immediate-early gene.
2. A vector for use in eukaryotic cell lines containing a DNA sequence comprising the promoter, enhancer and functionally complete 5'-untranslated region including the first intron of the human cytomegalovirus major immediate-early gene, wherein the 5'-untranslated region is linked directly to the DNA coding sequence of a heterologous gene.
3. A vector according to claim 2 wherein the vector includes a restriction site to allow the excision and/or insertion of the coding sequence of a heterologous gene.
4. A vector according to claim 1 or claim 2 wherein the human cytomegalovirus major immediate-early DNA includes a translation initiation signal.
5. A host cell transfected with a vector according to any of the preceding claims.
6. A process for the production of a heterologous polypeptide comprising culturing a host cell according to claim 4.
7. The use of a DNA sequence comprising the promoter, enhancer and functionally complete 5'-untranslated region including the first intron of the human cytomegalovirus major immediate-early gene for expression of a heterologous gene in a eukaryotic cell line wherein the human cytomegalovirus major immediate-early gene derived DNA is linked directly to the coding sequence of the heterologous gene.
8. A plasmid for use in a eukaryotic cell line comprising the human cytomegalovirus major immediate-early promoter and the functionally complete 5'-untranslated region of the major immediate-early gene, including the first intron, fused directly to a glutamine synthetase coding sequence at the translation initiation site.
9. A plasmid comprising the human cytomegalovirus major immediate-early promoter and the functionally complete 5'-untranslated region of the major immediate-early gene, including the first intron, fused directly to a tissue inhibitor of metalloproteinase (TIMP) coding sequence at the translation initiation site.
10. A plasmid comprising the human cytomegalovirus immediate-early promoter and the functionally complete 5'-untranslated region of the major immediate-early gene, including the first intron, wherein the 5'-untranslated region comprises a translation start site which is coincident with an NCO1 restriction site.

Patentansprüche

1. Vektor zur Verwendung in eukaryotischen Zell-Linien, der eine DNA-Sequenz, umfassend den Promotor, den Verstärker und die funktionell vollständige 5'-untranslatierte Region, inklusive des ersten

Introns, des größeren unmittelbar-frühen (major immediate-early) Gens des humanen Cytomegalovirus enthält, wobei die 5'-untranslatierte Region nicht direkt an die DNA-Codierungssequenz des natürlichen größeren unmittelbar-frühen Gens des humanen Cytomegalovirus gebunden ist.

- 5 2. Vektor zur Verwendung in eukaryotischen Zell-Linien, der eine DNA-Sequenz enthält, die den Promotor, den Verstärker und die funktionell vollständige 5'-untranslatierte Region, inklusive des ersten Introns, des größeren unmittelbar-frühen Gens des humanen Cytomegalovirus enthält, wobei die 5'-untranslatierte Region direkt an die DNA-Codierungssequenz eines heterologen Gens gebunden ist.
- 10 3. Vektor nach Anspruch 2, welcher eine Restriktionsstelle enthält, um die Excision und/oder Insertion der Codierungssequenz eines heterologen Gens zu gestatten.
4. Vektor nach Anspruch 1 oder Anspruch 2, wobei die größere unmittelbar-frühe DNA des humanen Cytomegalovirus ein Translationsinitiationssignal enthält.
- 15 5. Wirtszelle, die mit einem Vektor nach irgendeinem der vorhergehenden Ansprüche transfiziert ist.
6. Verfahren zur Herstellung eines heterologen Polypeptids, bei welchem Verfahren eine Wirtszelle nach Anspruch 4 gezüchtet wird.
- 20 7. Verwendung einer DNA-Sequenz, die den Promotor, den Verstärker und die funktionell vollständige 5'-untranslatierte Region, inklusive des ersten Introns, des größeren unmittelbar-frühen Gens des humanen Cytomegalovirus umfaßt, zur Expression eines heterologen Gens in einer eukaryotischen Zell-Linie, wobei die von dem größeren unmittelbar-frühen Gen des humanen Cytomegalovirus stammende DNA direkt an die Codierungssequenz des heterologen Gens gebunden ist.
- 25 8. Plasmid zur Verwendung in einer eukaryotischen Zell-Linie, das den größeren unmittelbar-frühen Promotor des humanen Cytomegalovirus und die funktionell vollständige 5'-untranslatierte Region des größeren unmittelbar-frühen Gens, inklusive des ersten Introns, direkt an eine Glutaminsynthase-Codierungssequenz an der Translationsinitiationsstelle fusioniert enthält.
- 30 9. Plasmid, das den größeren unmittelbar-frühen Promotor des humanen Cytomegalovirus und die funktionell vollständige 5'-untranslatierte Region des größeren unmittelbar-frühen Gens, inklusive des ersten Introns, direkt an die Codierungssequenz eines Gewebeinhibitors von Metalloproteinase (TIMP) an der Translationsinitiationsstelle fusioniert enthält.
- 35 10. Plasmid, das den größeren unmittelbar-frühen Promotor des humanen Cytomegalovirus und die funktionell vollständige 5'-untranslatierte Region des größeren unmittelbar-frühen Gens, inklusive des ersten Introns, enthält, wobei die 5'-untranslatierte Region eine Translationsstartstelle enthält, die mit einer NCO1-Restriktionsstelle zusammenfällt.
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Revendications

- 45 1. Vecteur pour utilisation dans des lignées cellulaires eucaryotes, contenant une séquence d'ADN comprenant le promoteur, l'activateur et la région non traduite en 5', fonctionnellement complète, comprenant le premier intron du gène précoce-immédiat majeur du cytomégalovirus humain, dans lequel la région non traduite en 5' n'est pas liée directement à la séquence codante de l'ADN du gène précoce-immédiat majeur du cytomégalovirus humain naturel.
- 50 2. Vecteur pour utilisation dans des lignées cellulaires eucaryotes, contenant une séquence d'ADN comprenant le promoteur, l'activateur et la région non traduite en 5', fonctionnellement complète, comprenant le premier intron du gène précoce-immédiat majeur du cytomégalovirus humain, dans lequel la région non traduite en 5' est liée directement à la séquence codante de l'ADN d'un gène hétérologue.
- 55 3. Vecteur selon la revendication 2; dans lequel le vecteur comprend un site de restriction pour permettre l'excision et/ou l'insertion de la séquence codante d'un gène hétérologue.

4. Vecteur selon la revendication 1 ou la revendication 2, dans lequel l'ADN précoce-immédiat majeur du cytomegalovirus humain comprend un signal d'initiation de traduction.
5. Cellule hôte transfectée avec un vecteur selon l'une quelconque des revendications précédentes.
- 5 6. Procédé pour la production d'un polypeptide hétérologue, comprenant la culture d'une cellule hôte selon la revendication 4.
7. Utilisation d'une séquence d'ADN comprenant le promoteur, l'activateur et la région non traduite en 5',
10 fonctionnellement complète, comprenant le premier intron du gène précoce-immédiat majeur du cytomegalovirus humain, pour l'expression d'un gène hétérologue dans une lignée cellulaire eucaryote, dans laquelle l'ADN provenant du gène précoceimmédiat majeur du cytomegalovirus humain est lié directement à la séquence codante du gène hétérologue.
- 15 8. Plasmide pour utilisation dans une lignée cellulaire eucaryote, comprenant le promoteur précoce-immédiat majeur du cytomegalovirus humain et la région non traduite en 5', fonctionnellement complète, du gène précoce-immédiat majeur, y compris le premier intron, soudée directement à une séquence codant pour la glutamine synthétase, au site d'initiation de traduction.
- 20 9. Plasmide comprenant le promoteur précoce-immédiat majeur du cytomegalovirus humain et la région non traduite en 5', fonctionnellement complète, du gène précoce-immédiat majeur, y compris le premier intron, soudée directement à une séquence codant pour l'inhibiteur tissulaire de métalloprotéi-nase (TIMP), au site d'initiation de traduction.
- 25 10. Plasmide comprenant le promoteur précoce-immédiat du cytomegalovirus humain et la région non traduite en 5', fonctionnellement complète, du gène précoce-immédiat majeur, y compris le premier intron, dans lequel la région non traduite en 5' comprend un site d'initiation de traduction qui coïncide avec un site de restriction NCO1.

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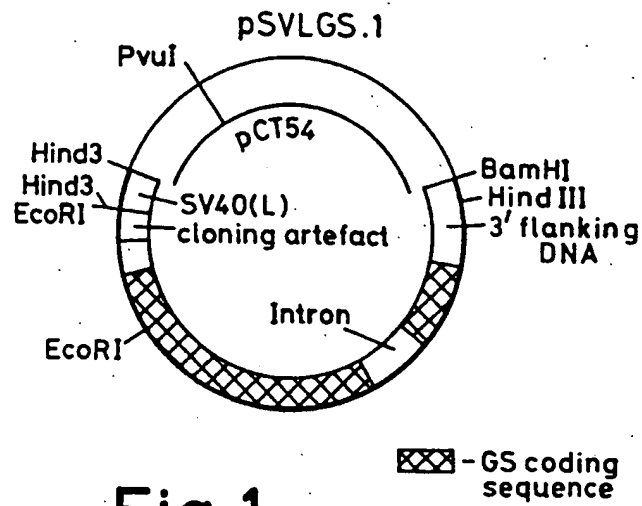


Fig. 1

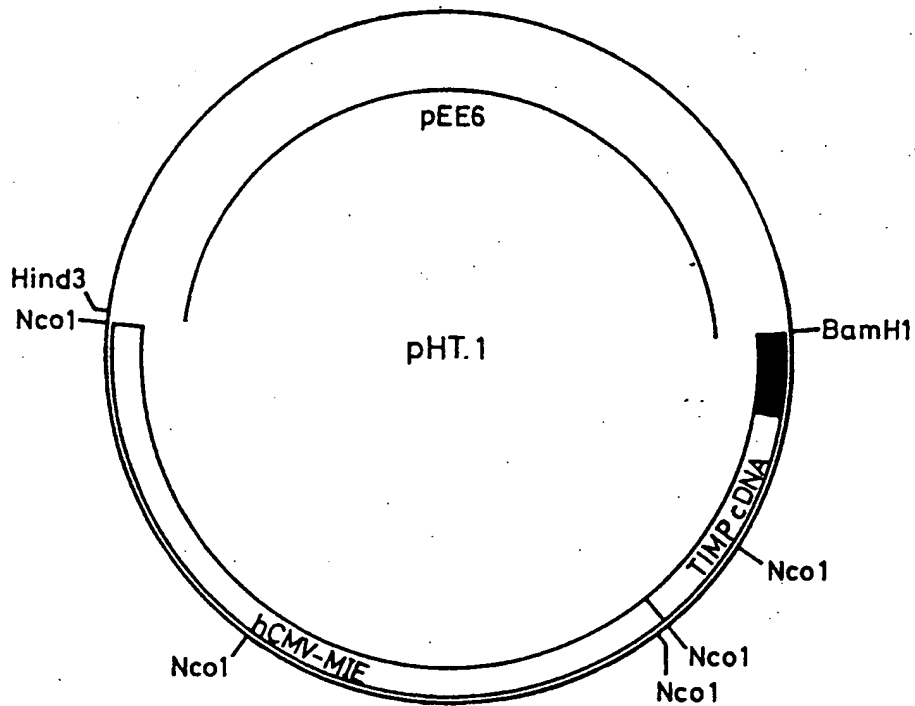


Fig. 2

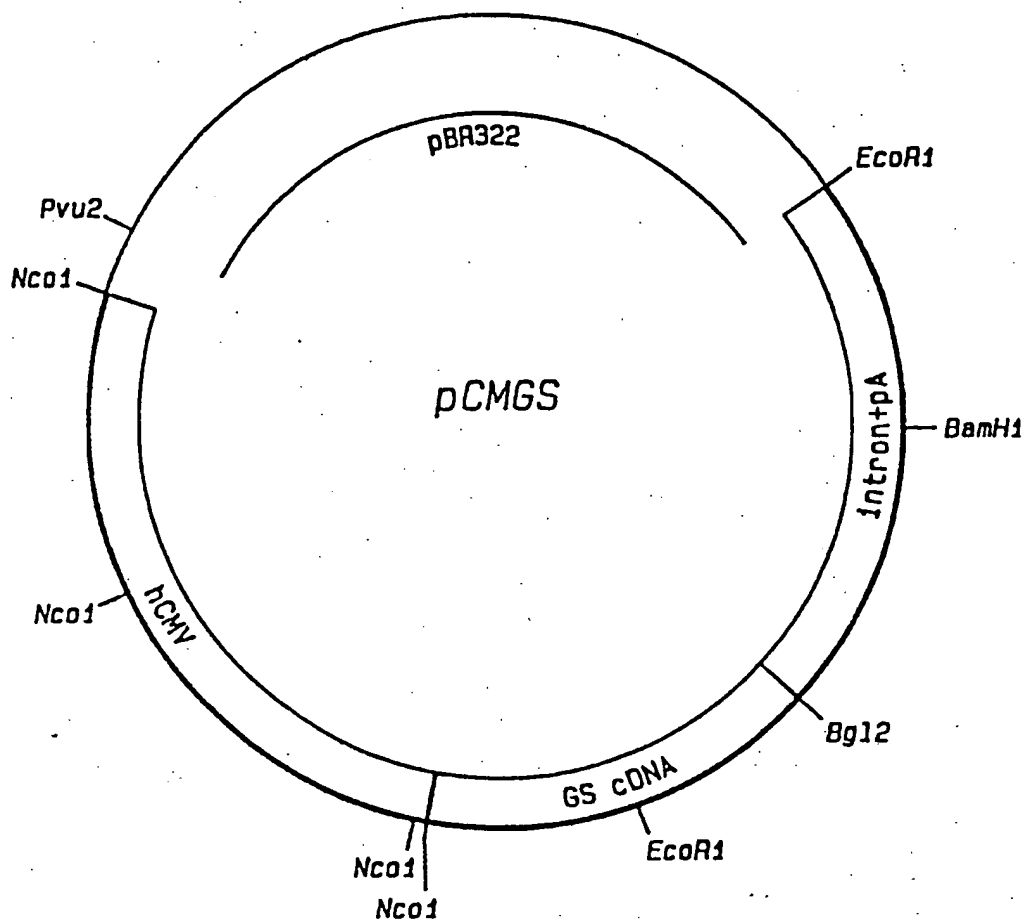


Fig. 3

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DNS          p          T          AM
sct          s          t          fl
aoy          t          3          lu
111          1          2          31
//          /

1  CCATGGTGTCAAGGACGGTGA CTGCACTGAATAATAAAATGTGTGTTTGTCCGAAATACG
   -----+-----+-----+-----+-----+-----+-----+-----+
60  GGTACCACAGTTCCTGCCACTGACGTCACTTATTATTTTACACACAAACAGGCTTTATGC

   CGTTTTGAGATTTCTGTGCGCCGACTAAATTCATGTCGCGCGATAGTGGTGTGTTTATCGCCG
61  -----+-----+-----+-----+-----+-----+-----+-----+
120  GCAAAACTCTAAAGACAGCGGCTGATTTAAGTACAGCGCGCTATCACCACAAATAGCGGC

           C
           l
           a
           1
121  ATAGAGATGGCGATATTGGA AAAATCGATATTTGAAAATATGGCATATTGAAAATGTGCG
   -----+-----+-----+-----+-----+-----+-----+-----+
180  TATCTCTACCGCTATAACCTTTT TAGCTATAAACTTTTATACCGTATAACTTTTACAGCG

           E
           c
           o
           r
           v

181  CGATGTGAGTTTCTGTGTA ACTGATATCGCCATTTTCCAAAAGTGATTTTGGGCATAC
   -----+-----+-----+-----+-----+-----+-----+-----+
240  GCTACACTCAAAGACACATTGACTATAGCGGTAAAAAGGTTTCTACTAAAAACCCGTATG

           E
           c
           o
           r
           v

241  GCGATATCTGGCGATAGCGGCTTATATCGTTTACGGGGGATGGCGATAGACGACTTTGGT
   -----+-----+-----+-----+-----+-----+-----+-----+
300  CGCTATAGACCGCTATCGCCGAATATAGCAAATGCCCCCTACCGCTATCTGCTGAAACCA

   GACTTGGGCGATTCTGTGTGTCGCAAATATCGCAGTTTCGATATAGGTGACAGACGATAT
301  -----+-----+-----+-----+-----+-----+-----+-----+
360  CTGAACCCGCTAAGACACACAGCGTTTATAGCGTCAAAGCTATATCCACTGTCTGCTATA

           C BH          N C
           f aa          s l
           r le          i a
           1 11          1 1
           /

361  GAGGCTATATCGCCGATAGAGGCGACATCAAGCTGGCACATGGCCAATGCATATCGATCT
   -----+-----+-----+-----+-----+-----+-----+-----+
420  CTCCGATATAGCGGCTATCTCCGCTGTAGTTCGACCGGTACCGGTTACGTATAGCTAGA

```

Fig. 4A

S C BH
 s f aa
 p r le
 1 1 11
 /

421 ATACATTGAATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCA
 -----+-----+-----+-----+-----+ 480
 TATGTAACTTAGTTATAACCGGTAATCGGTATAATAAGTAACCAATATATCGTATTTAGT
 S C BH
 s f aa
 p r le
 1 1 11
 /

481 ATATTGGCTATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTG
 -----+-----+-----+-----+-----+ 540
 TATAACCGATAACCGGTAACGTATGCAACATAGGTATAGTATTATACATGTAAATATAAC

H
 i M S
 n m p
 c e e
 2 1 1

541 GCTCATGTCCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAAT
 -----+-----+-----+-----+-----+ 600
 CGAGTACAGGTTGTAATGGCGGTACAACGTAACTAATAACTGATCAATAATTATCATT
 CAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGCGTTACATAACTTACGG
 601 -----+-----+-----+-----+-----+ 660
 GTTAATGCCCCAGTAATCAAGTATCGGGTATATACCTCAAGGCGCAATGTATTGAATGCC

B A A
 g h a
 l a t
 1 2 2

661 TAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCGCCCATTTGACGTCAATAATGACGT
 -----+-----+-----+-----+-----+ 720
 ATTTACCGGGCGGACCGACTGGCGGGTTGCTGGGGGCGGGTAACTGCAGTTATTACTGCA

A A
 h a
 a t
 2 2

721 ATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTAC
 -----+-----+-----+-----+-----+ 780
 TACAAGGGTATCATTGCGGTTATCCCTGAAAGGTAAGTGCAGTTACCCACCTCATAAATG

B N
 g d
 l e
 1 1

781 GGTAAGTGGCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTG
 -----+-----+-----+-----+-----+ 840
 CCATTTGACGGGTGAACCGTCATGTAGTTCACATAGTATACGGTTCATGCGGGGGATAAC

Fig. 4B

```

      A  A          B
      h  a          g
      a  t          l
      2  2          1
841  ACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACT
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 900
      TGCAGTTACTGCCATTTACCGGGCGGACCGTAATACGGGTCATGTACTGGAATACCCTGA

      S
      n          DNS
      a          sct
      B          aoy
      1          111
              //
901  TTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTT
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 960
      AAGGATGAACCGTCATGTAGATGCATAATCAGTAGCGATAATGGTACCACTACGCCAAAA

      GGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACC
961  -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1020
      CCGTCATGTAGTTACCCGCACCTATCGCCAAACTGAGTGCCCTAAAGGTTTCAGAGGTGG

      A  A          B
      h  a          a
      a  t          n
      2  2          1
1021 CCATTGACGTCAATGGGAGTTTGTGTTTGGCACCAAATCAACGGGACTTTCCAAAATGTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1080
      GGTAAGTGCAGTTACCCTCAAACAAAACCGTGTTTGTAGTTGCCCTGAAAGGTTTTACAG

      GTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
1081 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1140
      CATTGTTGAGGCGGGGTAAGTGCAGTTTACCCGCCATCCGCACATGCCACCCTCCAGATAT

      BH
      BssS
      apia
      n1Ac
      2211
      ///
1141 TAAGCAGAGCTCGTTTGTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTG
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1200
      ATTCGTCTCGAGCAAATCACTTGGCAGTCTAGCGGACCTCTGCGGTAGGTGCGACAAAAC

      B          N
      b          D BCGsSX
      v          s gfdpam
      2          a lriBca
              1 112223
              ////
1201 ACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1260
      TGGAGGTATCTTCTGTGGCCCTGGCTAGGTGCGAGGCGCGGCCCTTGCCACGTAACCTT

```

Fig. 4C

CGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCACCC
 1261-----+-----+-----+-----+-----+-----+ 1320
 GCGCCTAAGGGGCACGGTTCTCACTGCATTTCATGGCGGATATCTCAGATATCCGGGTGGG

B N
 Ss N sS
 tt s pp
 yX i Hh
 11 1 11
 /

CCTTGGCTTCTTATGCATGCTATACTGTTTTTGGCTTGGGGTCTATACACCCCGCTTCC
 1321-----+-----+-----+-----+-----+ 1380
 GGAACCGAAGAATACGTACGATATGACAAAACCGAACCCAGATATGTGGGGGCGAAGG

E
 S
 P
 1

TCATGTTATAGGTGATGGTATAGCTTAGCCTATAGGTGTGGGTATTGACCATTATTGAC
 1381-----+-----+-----+-----+-----+ 1440
 AGTACAATATCCACTACCATATCGAATCGGATATCCACACCCAATAACTGGTAATAACTG

P
 f
 l
 M
 1

CACTCCCCTATTGGTGACGATACTTTCCATTACTAATCCATAACATGGCTCTTTGCCACA
 1441-----+-----+-----+-----+-----+ 1500
 GTGAGGGGATAACCACTGCTATGAAAGGTAATGATTAGGTATTGTACCGAGAAACGGTGT

E
 C
 O
 S
 7

ACTCTCTTTATTGGCTATATGCCAATACACTGTCCTTCAGAGACTGACACGGACTCTGTA
 1501-----+-----+-----+-----+-----+ 1560
 TGAGAGAAATAACCGATATACGGTTATGTGACAGGAAGTCTCTGACTGTGCCTGAGACAT

E
 C
 O
 3
 1

TTTTACAGGATGGGGTCTCATTATTTATTATTTACAAATTCACATATACAACACCACGTCC
 1561-----+-----+-----+-----+-----+ 1620
 AAAAATGTCCTACCCCAGAGTAAATAATAAATGTTTAAAGTGATATGTTGTGGTGGCAGG

B X A A
 s h v f
 p o a 1
 1 2 1 3
 2

CCAGTGCCCGCAGTTTTTATTAAACATAACGTGGGATCTCCACGCGAATCTCGGGTACGT
 1621-----+-----+-----+-----+-----+ 1680
 GGTCACGGGCGTCAAAAATAATTGTATTGCACCCTAGAGGTGCGCTTAGAGCCCATGCA

Fig. 4D

Fig. 4E

[illegible]

Fig.4F

Fig. 5

The diagram illustrates the circular genome of Ee6hcmv (4718). The outer ring represents the genome, with various restriction enzyme sites marked around its circumference. The sites are labeled as follows (clockwise from the top): Cla1 < Sma1 < Sac1 < EcoR1 < Bcl1, Sty1 / Nco1 < Pst1 < Hind3 < BspM1 < Pst1 < Pvu1 < Xba1, Sca1 < BssH2 < BssH2, Hpa1, Af12 < Pvu2, Sac1, BspM2, P1M1, Sty1 < BstX1 < Nsi1 < Sph1, Bgl1 < Xma3 / Sac2, Sac1, SnaB1 < Sty1 / Nco1, Bgl1 < Nde1, Bgl1, Ssp1 < Bal1, Ssp1 < Bal1, Hind3 < Pst1, Bgl1, EE6HCMV, Bgl1, Ssp1, ApaL1 < EcoK, Xmn1, Sca1, Pvu1, Sph1 < Fsp1, Bgl1, Tth32 < Tth32, ApaL1, Acc1 < Sal1, Sph1, Naø1 < BamH1, Bsm1, Hpa1 < Bsm1, and POLYA. Internal features include a large arc labeled 'SAL-AVA' and a smaller arc labeled 'ampR'. A T7 promoter is indicated by a line labeled 'T7'. The genome size is noted as 4718.

INSERT HCMV PROMOTER FRAGMENT IN THE HIND3 SITE OF EES
Mismatch: 0 MinCuts = 1 MaxCuts: 3